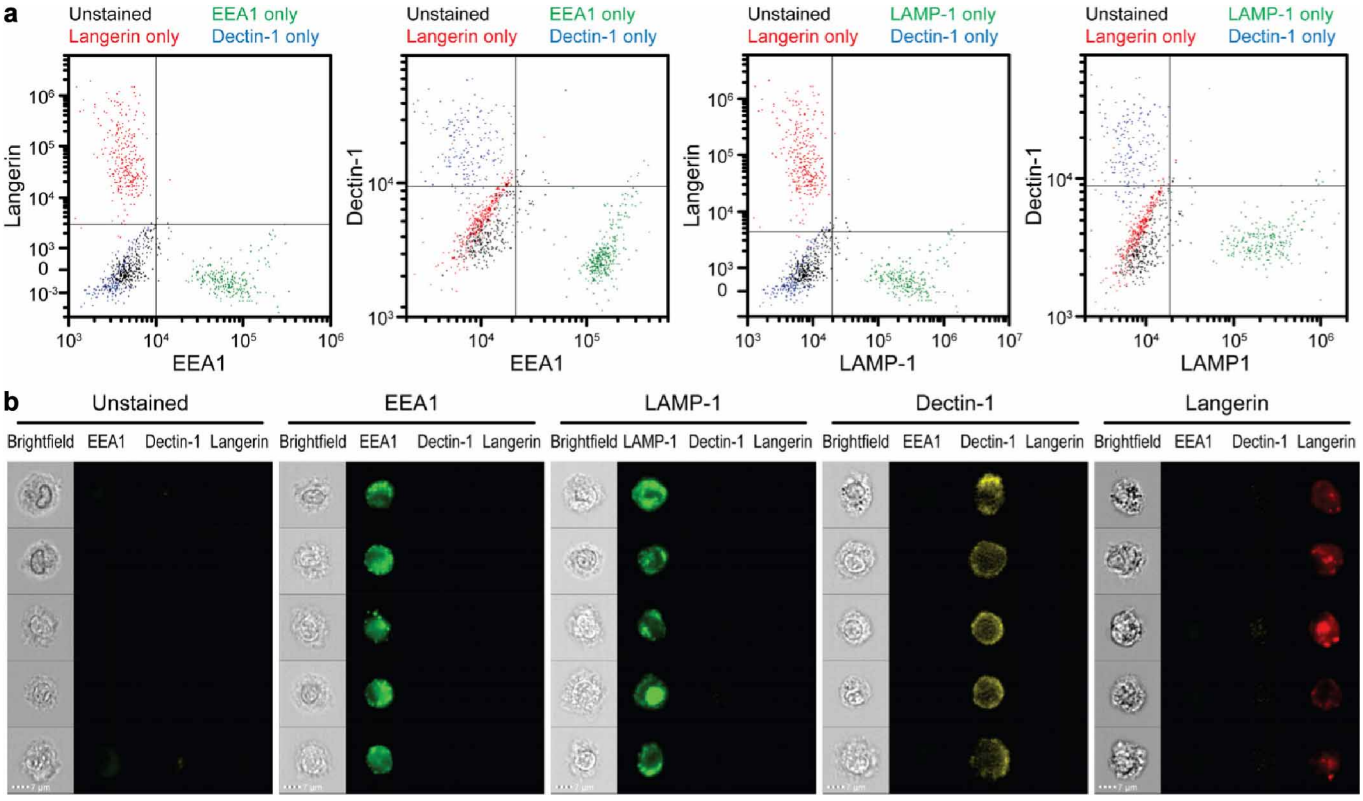
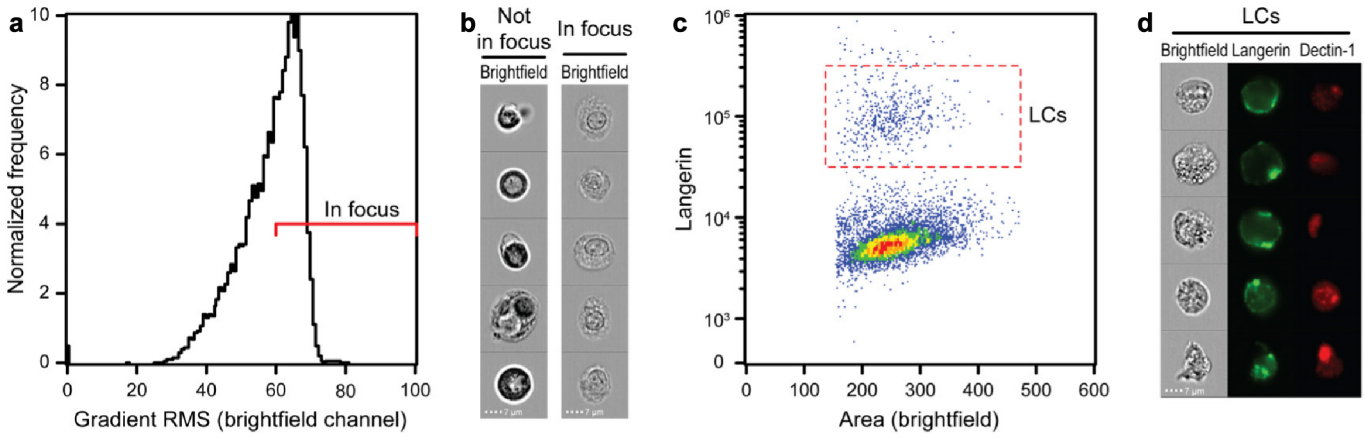
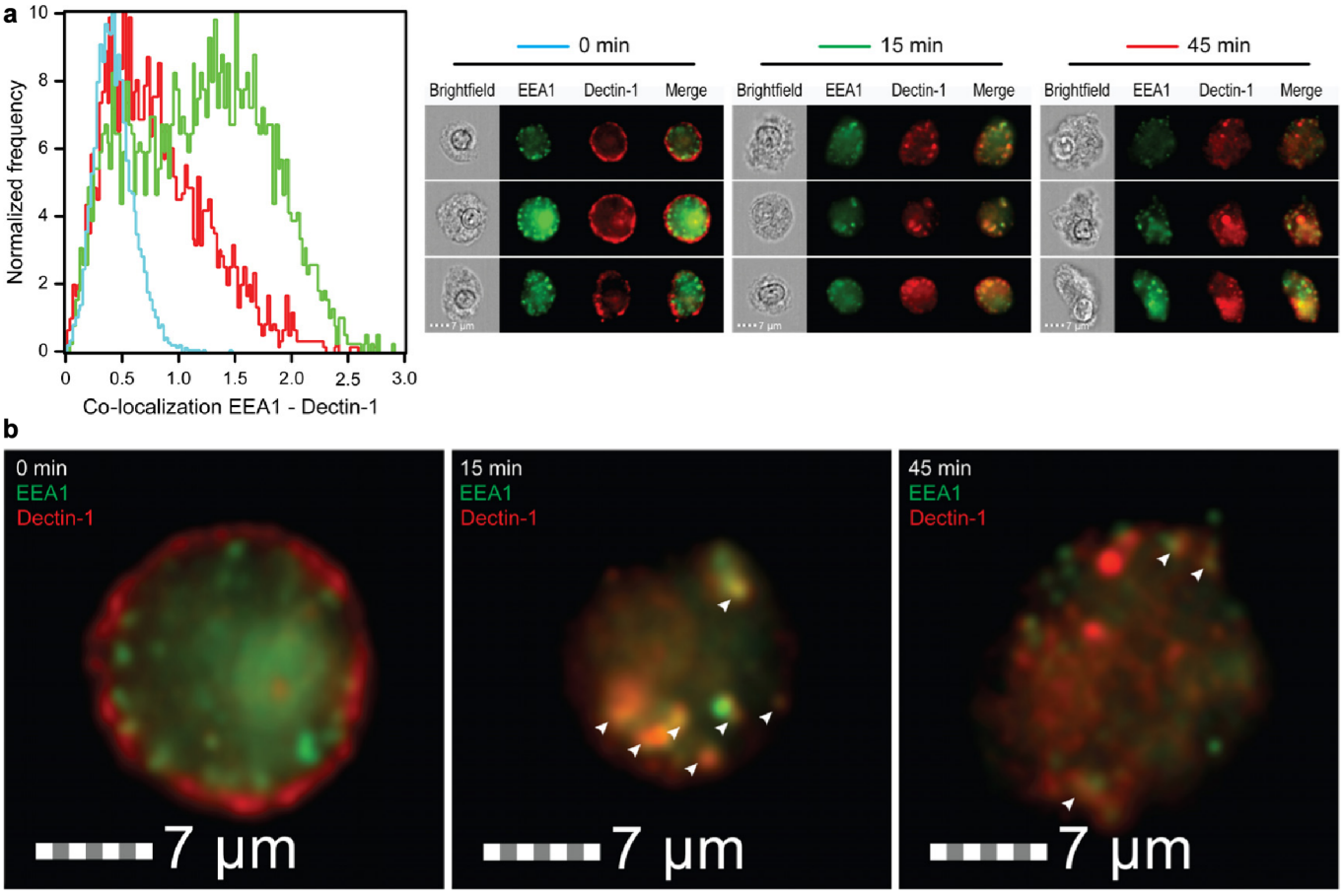


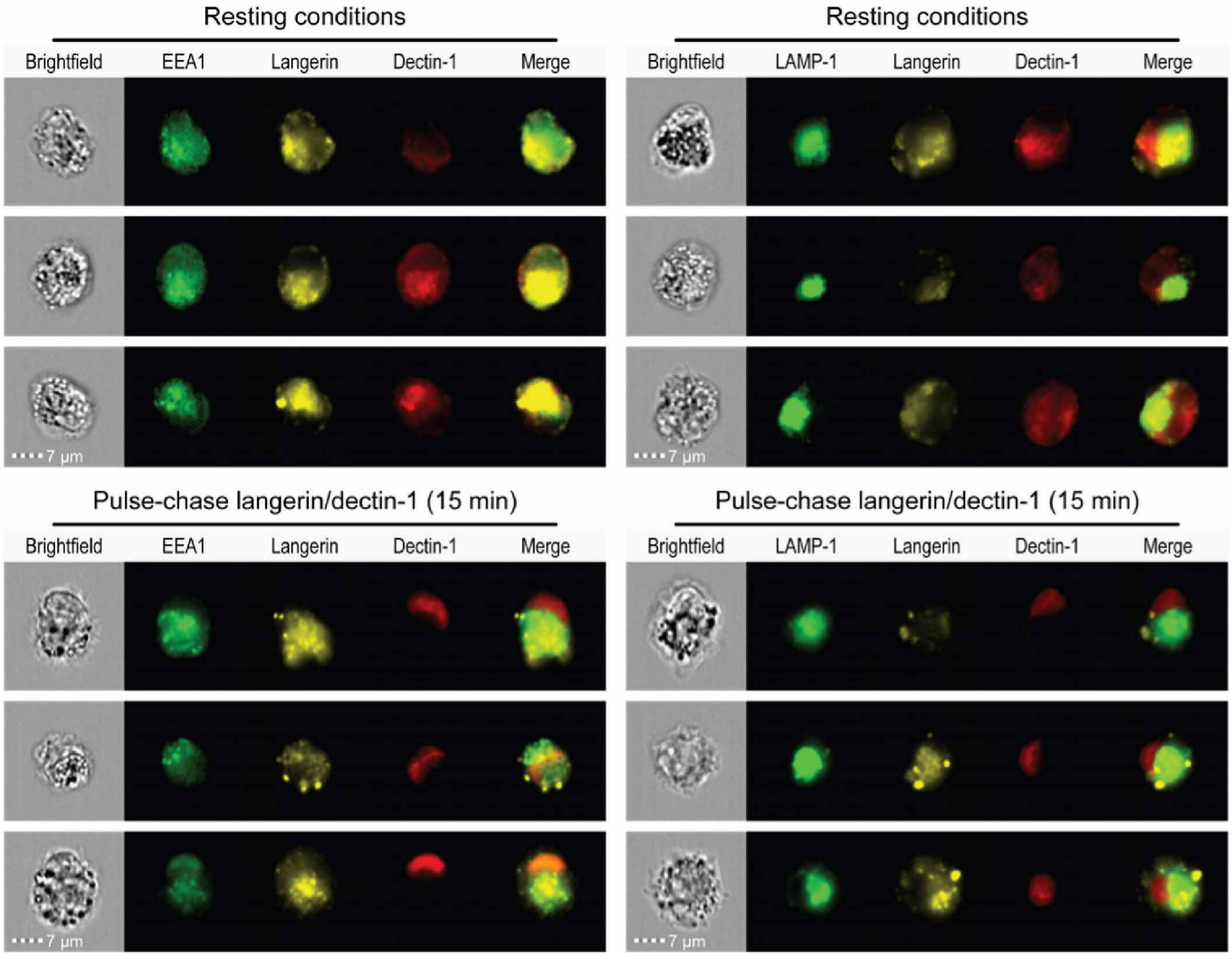
Supplementary Figure S1 Compensation in imaging flow cytometry experiments. A critical step in the analysis of imaging flow cytometry data consists of the elimination of fluorescence leakage in neighboring channels. This process is identical to compensation in classical flow cytometry and involves the acquisition of single-stained samples and the processing of the data with a compensation wizard present in the software package IDEAS (Amnis-Millipore). Once a compensation table has been generated, it can be applied to the raw data to process images without spectral overlap. In order to control that the compensation table has been generated flawlessly, it is applied to the single-staining controls and proper separation of individual channels is verified, as shown in (**a**). (**b**) Images were randomly selected from single-stained populations to verify that, indeed, only the expected channel contained fluorescence.



Supplementary Figure S2 Imaging flow cytometry analysis strategy. In imaging flow cytometry, images are taken from cells as they pass by the detector in a flow system. The objective is calibrated such that images are taken at the center of the flow. At the magnification used in the experiments reported in this manuscript (60×), the lateral resolution corresponds to approximately 300 nm, whilst the depth of focus is 2.5 μm. Therefore, cells that are slightly off the center of the flow may appear out of focus. In order to gate the cells that are properly focused, a necessary condition for further analysis, the *Gradient RMS* feature applied to the *Brightfield channel* was used, as depicted in (**a**). Gradient RMS measures the sharpness quality of images by detecting large changes (average gradient of a pixel normalized for variations in intensity levels) of pixel values in the image. (**b**) Examples of events according to their gradient RMS value. Only those events with values higher than 60, were further selected for analysis. (**c**) Since the amount of LCs was low and a minimum cell/rate is needed for proper acquisition, samples were spiked with fixed monocyte-derived dendritic cells. LCs could be gated by their high langerin expression. (**d**) Randomly selected LCs showing expression of langerin and dectin-1.



Supplementary Figure S3 Analysis of co-localization by imaging flow cytometry. *Bright Detail Similarity R3* is a feature designed to compare the small bright image detail of two images (pixel by pixel). Mathematically, *Bright Detail Similarity R3* is defined as the log transformed Pearson's correlation coefficient of the localized bright spots with a radius of three pixels or less within the masked area in the two input images. In practice, values higher than three are hardly ever obtained. To illustrate the range of values that can be obtained using this feature and their biological significance, the example of dectin-1 and EEA1 co-localization in monocyte-derived dendritic cells is provided. (**a**) Monocyte-derived dendritic cells were incubated with an anti-dectin-1 antibody (AF647-labeled) at 4°C for 30 min, washed, and chased for 0, 15, and 45 min at 37°C. Cells were then washed in ice-cold PBS, fixed in 4% paraformaldehyde, and further stained as described in the *Materials and Methods* section using the anti-EEA antibody (FITC-labeled). Cells were acquired by imaging flow cytometry as described in the *Materials and Methods* section and analyzed for EEA1 and dectin-1 co-localization using the feature *Bright Detail Similarity R3* applied to the EEA1 and the dectin-1 channels. Results clearly indicate a low co-localization at 0 min (approx. 0.5 ± 0.1), which increases dramatically after 15 min (approx. 1.2 ± 0.4), to then moderately decrease at time point 45 min (approx. 0.8 ± 0.2). Randomly selected images from each of the different time points demonstrate the absence of co-localization at start (with dectin-1 still clearly present at the cell membrane, while EEA1 shows its typical intracellular spotted distribution), a dramatic increase in fluorescence overlap after 15 min, which decreases at the last time point. (**b**) High-resolution images were selected to depict dectin-1^{+} endosomes in the merged images (white arrows). The number of endosomes is clearly superior at 15 min, correlating with the values obtained using the *Bright Detail Similarity R3* feature. Three-color co-localization can be interpreted in a similar way and is calculated using the *Bright Detail Co-localization 3* feature. Its typical values differ dramatically from 2-color co-localization and have a much lower range, hardly ever higher than 0.5.



Supplementary Figure S4 Representative images depicting 3-color co-localization. Images were randomly selected from the experiment as described in Figure 5f.